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Size-exclusion chromatographic NMR under HR-MAS

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Abstract

The addition of stationary phases or sample modifiers can be used to modify the separation achievable in the diffusion domain of diffusion NMR experiments or provide information on the nature of the analyte-sample modifier interaction.

Unfortunately, the addition of insoluble chromatographic stationary phases can lead to line broadening and a degradation in spectral resolution, largely due to differences in magnetic susceptibility between the sample and the stationary phase. High-resolution magic angle spinning techniques can be used to remove this broadening. Here, we attempt the application of HR-MAS to size-exclusion chromatographic NMR with limited success. Observed diffusion coefficients for polymer molecular weight reference standards are shown to be larger than those obtained on static samples. Further investigation reveals under HR-MAS it is possible to obtain reasonably accurate estimates of diffusion coefficients, either using full rotor synchronisation or sophisticated pulse sequences. The requirement for restricting the sample to the centre of the MAS rotor to ensure homogeneous magnetic and RF fields is also tested.

Introduction

Nuclear magnetic resonance spectroscopy is one of the key analytical techniques for the elucidation or verification of molecular structure.^[1, 2] There are a huge number of experiments available in the spectroscopic toolbox allowing an atomic level of detail to be obtained.^[1, 2] However, the vast majority of these experiments are best performed on pure samples, the presence of impurities or mixtures can lead to ambiguity in the analysis and interpretation. This spectral complexity can be overcome by separating the mixture, either using traditional chromatographic methods, whether on- or offline,^[3, 4] or by pseudoseparation using some molecular parameter such as diffusion coefficient^[5] or the generation of a maximum-quantum spectrum.^[6] The use of diffusion coefficient to separate mixtures is well documented,^[5, 7] with the ability to resolve molecules with reasonably small differences in diffusion coefficient under favourable conditions, however, the technique is limited in cases of spectral overlap.^[5, 7] In 2003 Caldarelli and co-workers proposed a method to improve resolution in the diffusion dimension by the addition of a silica stationary phase normally used in High Performance Liquid Chromatography (HPLC).^[8-10] They showed that separation was improved and correlated with the degree of interaction between the analyte and stationary phase as predicted by traditional chromatographic models.^[11, 12] The idea of adding a sample modifier has been proposed and utilised by a number of groups and is known as both chromatographic NMR^[9] or Matrix-Assisted DOSY (MAD).^[13, 14] Typical sample modifiers include: bare and functionalised silica,^[8-10, 15, 16] polymers,^[17-19] nanoparticles,^[20, 21] surfactant micelles^[13, 22, 23] and chiral shift reagents.^[14]

Recently we have extended the concept of chromatographic NMR to the use of size-exclusion stationary phases.^[24-26] These phases comprise a porous material, typically based on cross-linked dextran, with pore sizes on the order of 10-100 nm.

Chromatographic separation depends on the time different analytes spend exploring the pores, with smaller molecules typically spending longer inside the pores than larger molecules.^[27, 28] While size-exclusion phases do not offer an improvement in diffusion resolution, as smaller molecules are retarded to a greater degree than larger molecules,^[27] the interaction between the analyte and stationary phase, and therefore the change in observed diffusion coefficient upon addition of the stationary phase, can be interpreted in terms of size exclusion effects^[24, 26] and provide information on the nature of the interaction between the stationary phase and the analyte.

Several of the common sample modifiers proposed for chromatographic NMR result in additional line broadening being observed, limiting the spectral resolution. This line broadening arises as a result of magnetic susceptibility differences between the particles of the stationary phase and the bulk solvent.^[8, 9, 15, 29] Two approaches have been proposed to alleviate this line broadening and restore high resolution in the spectral dimension, in addition to the improved resolution in the diffusion dimension. The first is magic angle spinning, typically only moderate speeds of around 2-4 kHz are required.^[8-10] Under these conditions, liquid-like line shapes are restored at the cost of the appearance of spinning side bands and some concern over the accuracy of the measured diffusion coefficient due to effects such as sample vortexing.^[10, 30] The second approach to reducing the susceptibility-induced line broadening is to match the magnetic susceptibility of the solvent to that of the stationary phase.^[15] This is typically done in an empirical manner, adjusting the solvent composition to minimise

the spectral line width.^[15] This approach is, however, limited as the stationary phase must remain stable in the solvent mixture used. This is possible for silica stationary phases which are able to tolerate a wide range of solvents, but more of a challenge for dextran, poly(acrylamide) or other cross-linked polymer-based stationary phases and impractical for sample modifiers based on surfactants.

In order to improve the spectral resolution in size-exclusion chromatographic NMR, in this paper we therefore employ high-resolution magic angle spinning. We present some preliminary measurements of diffusion coefficients under HR-MAS with size-exclusion stationary phases which reveal some issues with this technique. Further investigation into the role of pulse sequence design and timings, including the unreliable measurement of the diffusion coefficient under certain conditions,^[10, 30] demonstrates that it is possible to obtain reliable diffusion measurements under HR-MAS conditions. We note in passing a bug in the vendor-supplied pulse sequences which hindered our analysis.

Materials and Methods

Sample Preparation

Poly(styrene sulfate) molecular weight reference standards (PDI < 1.20) were purchased from Kromatek (Essex, UK) as used as obtained. Deuterium oxide and benzene-*d*₆ were purchased from either Sigma Aldrich (Dorset, UK) or Goss Scientific (Cheshire, UK). All other chemicals were purchased from Sigma Aldrich. The polymers, with weight-average molecules weights of 10.6, 14.9, 20.7, 32.9 and 63.9 kDa, were prepared as 0.2 mM solutions in a buffer system comprising 150 mM sodium chloride and 50 mM sodium phosphate at pH 9. Sephadex G-50 stationary

phase was swelled at a concentration of 60 mg mL⁻¹ in the polymer solution for a minimum of three hours. The stationary phase suspension was then shaken and 80 µL were transferred to a 4 mm OD zirconia rotor. The rotor was held vertically and the suspension was left to settle under gravity. The supernatant (40 µL) was then removed before closing the rotor with the cap and drive ring.

NMR Spectroscopy

All NMR experiments were performed using a Varian VNMRs600 (Agilent Technologies, Yarnton, UK) operating at an ¹H frequency of 599.6 MHz. HR-MAS spectra were collected using a 4 mm gHX-Nano probe equipped with a magic angle gradient coil capable of producing gradients of approximately 1.66 T m⁻¹ along the sample rotation axis. The sample temperature was regulated to be 298 K as measured by the VT controller. The actual sample temperature under HR-MAS conditions (ν_r = 2 kHz) was 305 K measured using a ethylene glycol thermometer. Non-spinning spectra were recorded using a 5 mm X{¹H} probe with an actively shielded z-gradient coil capable of approximately 0.7 T m⁻¹. For these spectra the temperature was regulated at 298 K.

Diffusion measurements were principally performed using the Gradient Compensated STimulated Echo (GCSTE) , BiPolar Pulse STimulated Echo (BPPSTE)^[31] or Oneshot^[32] sequences as provided in the Agilent DOSY tools package. Typical parameters were as follows: a diffusion labelling period of 50-100 ms, diffusion labelling gradients were 1-3 ms in duration and comprised 15 intensity points equally spaced in g^2 from 0.0457 to 0.8129 T m⁻¹. Spectra were acquired over a spectral window of 9615.4 Hz using 9615 complex data points. All spectra were processed

using the NMR plugin of Mestrenova (Santiago de Compostela, Spain) or DOSY Toolbox^[33] as appropriate, with 5 Hz exponential line broadening prior to Fourier transformation and subsequent baseline correction with a second order polynomial. Diffusion data were fitted using a single exponential function to the Stejskal-Tanner equation, suitably modified for the appropriate pulse sequence.^[31, 32]

Results and Discussion

To demonstrate the effect addition of a stationary phase has on spectral quality and the improvement under HR-MAS conditions, Figure 1 shows the ¹H NMR of a 63.9 kDa sample of poly(styrene sulfonate) (PSS). Addition of the stationary phase causes an increase in the observed line width, along with a degradation in spectral resolution, and the appearance of signals in the 3-4 ppm range arising from the dextran backbone of the stationary phase. Repeating the experiment under HR-MAS at a spin rate of 2 kHz results in a return to the liquid-like line shape observed in the absence of the stationary phase, but with the added complication of spinning sidebands, indicated by * and ** for the solvent and stationary phase respectively. Given the relatively slow spinning speeds used in HR-MAS these sidebands will always occur within the spectral window, however, they can be moved away from signals of interest by small adjustments of the spinning rate.

Previous work has utilised size-exclusion stationary phases in standard 5 mm NMR tubes under static conditions, with the associated reduction in spectral resolution.^[24, 26] Performing similar experiments using polymer molecular weight reference standards under HR-MAS with and without a Sephadex G-50 stationary phase results in the measured diffusion coefficients presented in Figure 2. The error bars show that there

is an uncertainty of 10-25% in the measured diffusion coefficient, partially as a result of low signal to noise in the experiment and the potential overlap of spinning sidebands arising from the Sephadex stationary phase. For comparison, the same measurements performed on static samples (in a 5 mm tube) are also shown.^[24] There are clearly alarming differences between the measurements recorded under magic angle spinning compared to those reported previously. In the absence of the stationary phase, the measured diffusion coefficients obtained under HR-MAS appear to be approximately a factor of 2 larger than those obtained in the static samples. Differences in observed diffusion coefficient between spinning and static sample have been reported previously in the case of low viscosity solvents.^[10] A possible initial explanation is that the act of spinning the sample generates bulk sample motion, such as vortexing, resulting in erroneous diffusion coefficients being returned by the analysis. The trends in the measured diffusion coefficients are, however, similar between the spinning and static cases. Fitting these data to the phenomenological equation, similar to that obtained by Anderson and Stoddart^[34, 35] and Determann and Michel^[36], used previously:^[24, 26]

$$\log M = a_0 - a_1 D \quad (1)$$

results in the parameters given in Table 1. The similarity of the parameters for the spinning and static samples suggests that the polymer reference standards are behaving in a comparable manner under both sets of experimental conditions albeit with different measured diffusion coefficients.

On addition of the Sephadex G-50 stationary phase there is a further marked change in the measured diffusion coefficients. This change is an unexpected *increase* in the diffusion coefficient. Previous experiments with size exclusion phases,^[25, 26] resulted

in a decrease in measured diffusion coefficient due to molecules entering the pores of the stationary phase and being retarded, with the degree of retardation being dependent on both the molecular weight and pore size.^[27] Chromatographic NMR of benzene and silica stationary phases has shown an increase in diffusion coefficient upon addition of the stationary phase. This was rationalised by including the vapour phase of the benzene in a condensation-evaporation equilibrium between the solvent and stationary phase.^[37] This mechanism is unlikely to be operative in the case of the polymers reported here due to their low vapour pressure. The results shown in Figure 2 clearly indicate that in its current form, the simple transfer of the size-exclusion chromatographic NMR method to HR-MAS is not possible. There are clearly factors arising both from the application of magic angle spinning and combination with the stationary phase which require further investigation.

In order to understand further the influence of MAS on the diffusion experiments a series of measurements were performed using three diffusion experiments of increasing sophistication: the gradient-compensated stimulated echo (GCSTE), the bipolar pulse stimulate echo (BPPSTE)^[31] and the Oneshot experiment,^[32] monitoring the residual HOD signal of a D₂O sample. Diffusion measurements were performed as a function of spin rate, and with varying lengths of diffusion encoding gradient (δ). The results are shown in Figures 3(a), (c) and (e) for the three pulse sequences considered. It is immediately clear that for the GCSTE and BPPSTE sequences there is strong variation on the observed diffusion coefficient with spin rate and the duration of the diffusion encoding gradient. For example, with the GCSTE experiment shown in Figure 3(a) there is wild variation in the observed diffusion coefficient over an order of magnitude. A similar pattern, though less severe, is seen

for the BPPSTE sequence in Figure 3(c). The Oneshot sequence shows broadly similar diffusion coefficients across all experimental parameters tested, with a variation of only around 10% of the true diffusion coefficient for D₂O ($D = 2.3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$).^[38] To ensure the best possible accuracy in the measured diffusion coefficient, it is important that the gradient pulses used for diffusion encoding and decoding excite the same spin packet and therefore should be synchronised to the rotor period, both in their duration and interpulse timing.^[30, 39, 40] Viel et al. suggest, however, that this may not be necessary, reporting that no such synchronisation was employed in their measurements.^[10] In the standard implementation in the Agilent library, the total area of the diffusion encoding gradients are rotor-synchronised, but other parameters, including the interpulse spacing Δ are not synchronised with the sample rotation. Therefore the diffusion decoding gradient can end up being applied at a different point of the rotor cycle compared with the corresponding diffusion encoding gradient. Figures 3(b), (d) and (f) show the results of repeating the experiments in Figures 3(a), (c) and (e) ensuring that the timings of all RF-pulses, gradient durations and delays are synchronised with the sample rotation. Clearly differences with and without rotor synchronisation are apparent for the GCSTE (Figures 3(a) and (b)) and BPPSTE sequences (Figures 3(c) and (d)), but not for the Oneshot experiment (Figures 3(e) and (f)). Rotor synchronisation of the gradient pulses and delays appears to be imperative for the GCSTE and BPPSTE sequences, but less important for the Oneshot experiment. There are clearly some issues with the BPPSTE sequence, especially with long gradient pulses, however, the performance is clearly improved compared with no rotor synchronisation. The BPPSTE sequence incorporates bipolar gradient pulses which significantly reduce the impact of any gradient induced eddy currents.^[31] The Oneshot sequence includes a unbalancing factor to allow unwanted coherences to be

destroyed by the diffusion encoding gradients, negating the need for extensive phase cycling.^[32] The Oneshot sequence is an improved version of the BPPSTE sequence and therefore it is unsurprising that the two sequences produce similar results. The similarity in the results obtained between the rotor-synchronised BPPSTE and the Oneshot sequence, with or without rotor synchronisation, suggest that the improvements in the Oneshot sequence over the BPPSTE remove some of the additional complications which are partially ameliorated by the rotor synchronisation. We note that there is a bug in the original Agilent-supplied versions of the GCSTE, BPPSTE and Oneshot sequences. Data obtained from these sequences, when processed with VnmrJ or DOSYToolbox resulted in diffusion coefficients which are a function of both spin rate and gradient duration. Further details are in supplementary information.

A previous demonstration of chromatographic NMR under HR-MAS by Caldarelli and coworkers also showed that there were differences in the observed diffusion coefficient between static and spinning samples.^[10] In this case the differences were attributed to mechanical mixing and vortexing effects, with the influence of sample geometry also an important factor: restricting the sample volume to the regions of high gradient linearity lead to diffusion coefficients for small molecules in good agreement with literature data.^[10] Small intensity fluctuations in the echo attenuation curve have also been reported in experiments performed with similar sample geometry to that used here.^[30] These fluctuations are reported to arise from vortexing of the sample and can be mostly removed by extensive signal averaging.^[30] The effect of linear (coherent) motions can also be reduced by the use of convection compensated sequences,^[41] however, for the systems reported here little improvement

is seen (data not shown), likely due to the increased pulse sequence complexity. Restricting the sample diameter has the added benefit of reducing the exposure to radial variation in the B_1 rf -field which will be modulated by the MAS due to the lack of cylindrical symmetry in a solenoid coil.^[42] This modulation of the B_1 rf -field has been shown to be the source of signal loss in TOCSY experiments performed under HR-MAS conditions.^[42, 43] Slow sample spinning (~ 20 Hz) has also been suggested a method for mitigating against the effects of sample convection in diffusion NMR experiments,^[44] however, convection effects have recently been reported to be more insidious than previously thought.^[45] Bakhmutov has also shown that MAS can lead to changes in observed signal behaviour for spin-lattice relaxation measurements in liquid samples. A change from single to biexponential behaviour was observed as a function of spin rate, and attributed to the formation of an air bubble, held concentrically along the rotor axis by centrifugal forces, causing increased paramagnetic relaxation at the interface.^[46]

Following the arguments of Viel et al.^[10] and restricting the sample volume should reduce the dependence of the measured diffusion coefficient on spin rate by restricting the active volume to the centre of the sample rotor. Two sample restriction modalities were employed, the first was designed to restrict the sample geometry radially, i.e. closer to the rotation axis, and reduce the effects of radial field inhomogeneities^[39] and vortexing of the sample,^[10, 30] while the second restricted the sample dimensions axially to regions of greater gradient linearity.^[10] To achieve radial restriction a cylindrical Teflon spacer was introduced into the rotor to reduce the sample to a diameter of 1.5 mm, a reduction of 53%. The measured diffusion coefficient of HOD as a function of spin rate under these conditions for the Oneshot

sequence are shown in Figure 4(a). These data show almost identical trends to those in Figure 3(c), with slight overestimations of the diffusion coefficient at slow spinning rates. Axial restriction was performed by including a 1 mm thick Telfon disc (3.1mm diameter) at the top and bottom of the sample volume. This reduces the active sample volume by 43%. The results of these measurements are presented in Figure 4(b).

These again show very similar profiles to those in Figure 3(c) and 4(a). Combining the two approaches, i.e. utilising a sample which is restricted both axially and radially gives the results shown in Figure 4(c). In this arrangement the sample volume is approximately 4.7 μL , restricted to the centre of the rotor. Very similar trends in measured diffusion coefficient as a function of spin rate or duration of the diffusion encoding gradient are seen, with a slight minor deviation for higher spin rates and short gradient pulse duration. Taken together, these results suggest that restricting the sample to the centre of the rotor, either radially or axially, is not required for solvents of moderate viscosity, such as D_2O , and that minor inconsistencies in the measured diffusion coefficient are likely to arise from vortexing of the sample in the manner described previously.^[10]

In addition to providing spectral simplification, spinning a sample at high speed under MAS conditions also has the potential to introduce other physical effects as a result of the large mechanical forces present. The acceleration produced at the inside wall of a MAS rotor can be considerable.^[47] Even in the case of the 4 mm rotor used here, with an internal diameter of 3.14 mm, at the moderate speed of 2 kHz the acceleration at the rotor wall is over $20,000 \times g$. Bertini and co-workers have demonstrated that under fast spinning rates, large biomolecules can be sedimented to the rotor walls akin to ultracentrifugation.^[47-49] Figure 5 shows the concentration profile for the PSS

samples used in the 4 mm HR-MAS rotor at a spinning speed of 2 kHz, calculated using the approach of Bertini et al.^[48-50] While this does not show complete sedimentation of the polymers to the rotor wall, it is clear that there is a non-uniform concentration profile across the rotor, with increased concentration at the rotor edge and a concomitant decrease at the rotor centre. The difference in concentration is as much as a factor of >2 for the larger polymer standards. The size-exclusion stationary phase, when present, will also be exposed to the same physical forces and having a much larger effective molecular weight due to crosslinking, therefore likely to be predominantly found in the outer portions of the rotor. The result of this sedimentation effect is that the loading of the polymer solution into the stationary phase will be considerably different compared to the static case reported previously.^[24, 26] The loading of the stationary phase, i.e. the ratio of solution to stationary phase, has been shown to have a dramatic effect on the modulation of the diffusion coefficient caused by a given stationary phase.^[37] This effect is postulated to depend on whether mass transport is confined just to the intraparticle pores or whether there is sufficient solvent to allow escape into the interparticle space.^[11, 37] In the case of the samples used here, the ratio of solution to stationary phase is high, therefore the polymers are able to explore both the intra- and interparticle voids. Under the influence of MAS, sedimentation effects will therefore significantly distort the distribution of both the stationary phase and polymer, and hence the stationary phase loading, radially across the rotor. This spatial variation in the sample under MAS conditions may then lead to a distribution of diffusion modulation effects upon addition of the stationary phase should there be any sample vortexing present.

Conclusions

The addition of a chromatographic stationary phase to an NMR sample has the potential for great utility in improving the diffusion resolution^[8, 9, 14, 15, 23] or provide information on the analyte-stationary phase interaction.^[12, 37] However, the presence of an insoluble component in the sample can have deleterious effects on the spectral quality.^[10, 15] The use of HR-MAS methods to reduce these effects^[10] has been applied here in the context of size-exclusion chromatographic stationary phases, however, with unexpected results. Using size-exclusion chromatographic stationary phases under HR-MAS yields unexpected results in that the observed diffusion coefficient is larger in the presence of the stationary phase. Evaporation-condensation, postulated previously for benzene-silica systems,^[11] is unlikely to be responsible. We confirm that it is possible to obtain reliable estimates of the diffusion coefficient under HR-MAS conditions using either rotor synchronisation of the gradient pulses and delays,^[30, 39, 40] or more sophisticated pulse sequences such as Oneshot.^[32] The discrepancies in observed diffusion coefficients using SEC phases are currently under further investigation.

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Tables and Table Captions

Table 1: Parameters returned from fitting eq 1 to the diffusion coefficient data presented in Figure 2.

Sample	a_0	$a_1 / 10^{10} \text{ s m}^{-2}$	R^2
PSS standards – static ^[24]	5.11	1.50	0.96
PSS standards – static + Sephadex G50 ^[24]	5.30	2.52	0.93
PSS standards – 2 kHz MAS	5.69	1.25	0.81

Figure Captions

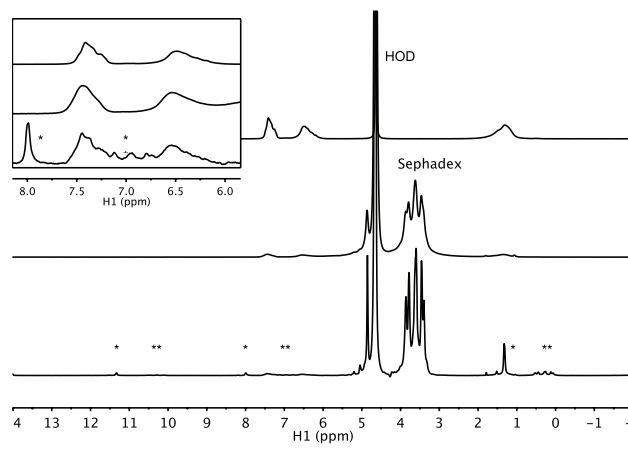
Figure 1: ^1H NMR spectra of 63.9 kDa poly(styrene sulfonate). The upper trace is PSS only, middle trace is in the presence of Sephadex G-50 and the lower trace is under 2 kHz HR-MAS. * and ** mark spinning sidebands arising from the HOD and Sephadex G-50 signals respectively.

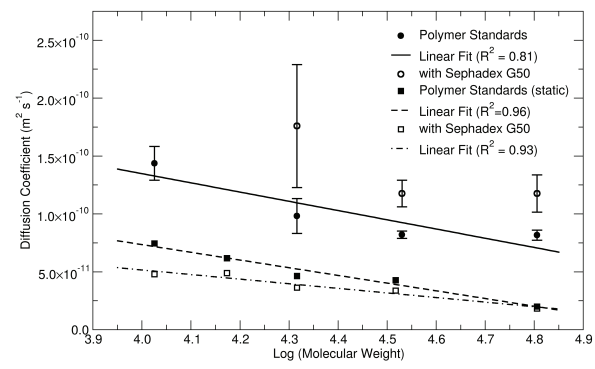
Figure 2: Measured diffusion coefficient as a function of $\log(\text{molecular weight})$ for the poly(styrene sulfonate) reference standards under HR-MAS conditions ($\nu_r = 2$ kHz) with and without Sephadex G-50. For comparison, diffusion coefficients for the same PSS samples under static conditions, in the absence of stationary phase,^[24] are also shown.

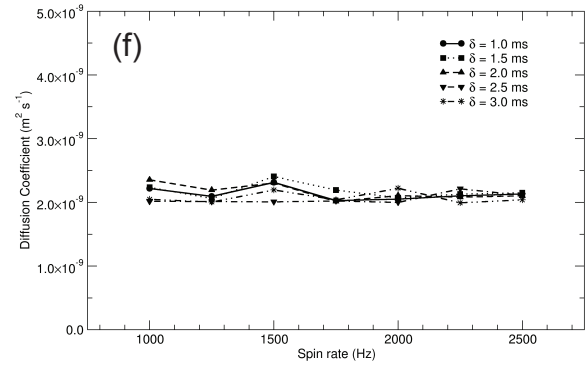
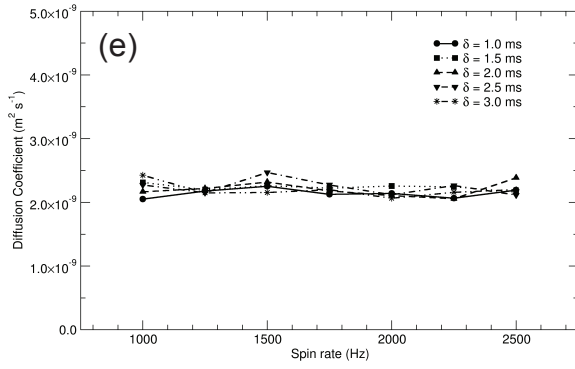
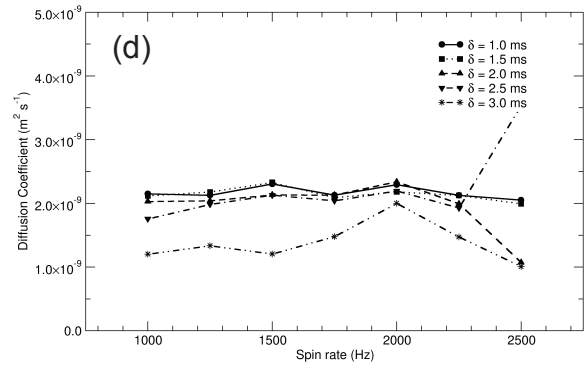
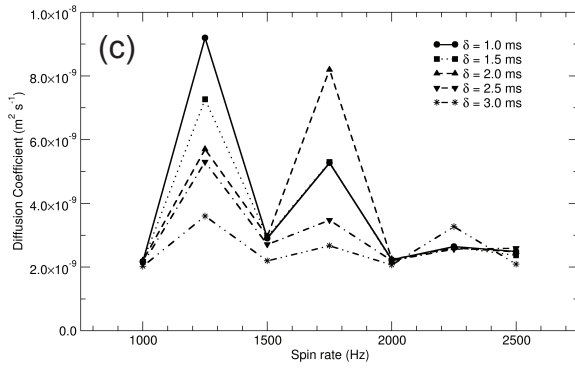
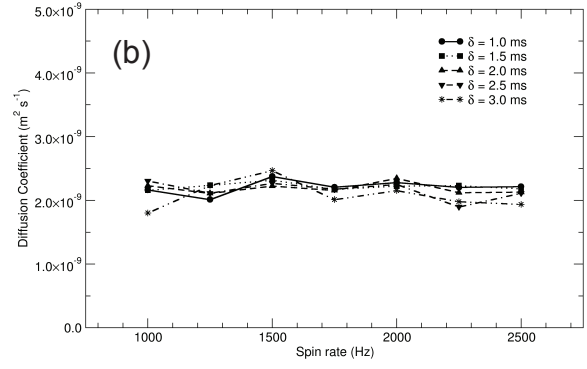
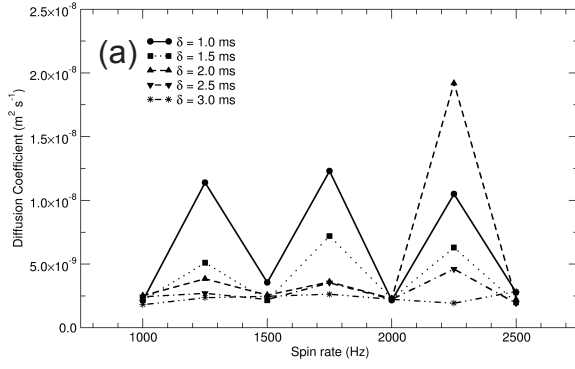
Figure 3: Observed variation in the measured diffusion coefficient of HOD with spin rate using a diffusion encoding time of Δ of 100 ms. (a) and (b) are for the gradient compensated stimulated echo (GCSTE), (c) and (d) for the bipolar pulse stimulated echo (BPPSTE) and (e) and (f) for the Oneshot sequence. (a) (c) and (e) are the pulse sequences as supplied in the Agilent library while (b), (d) and (f) utilise complete rotor synchronisation of the RF-pulses, gradient durations and delays.

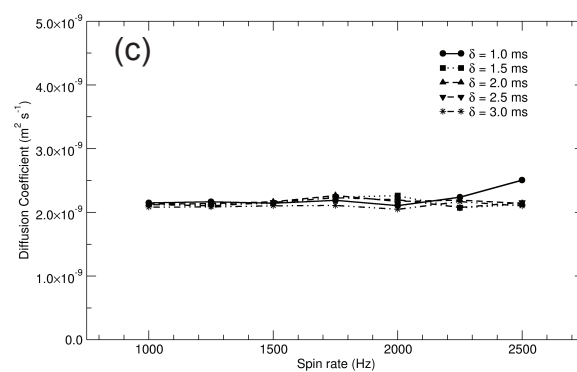
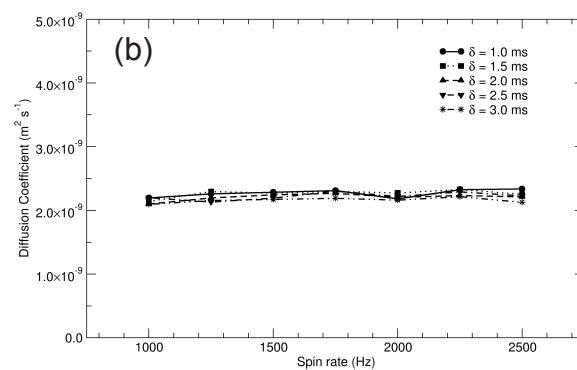
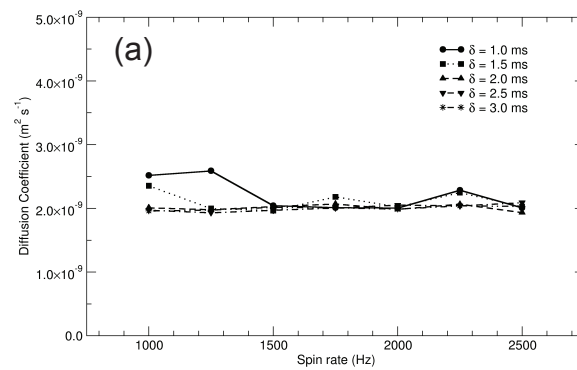
Figure 4: Observed variation in the measured diffusion coefficient of HOD with spin rate recorded using the Oneshot sequence and a diffusion encoding time Δ of 100 ms. (a) the sample was confined to a diameter of 1.5 mm by the inclusion of a Teflon spacer, (b) the sample was restricted to a height of 2.6 mm using a pair of 1 mm Teflon discs above and below the sample. (c) Combination of both sample restriction methods.

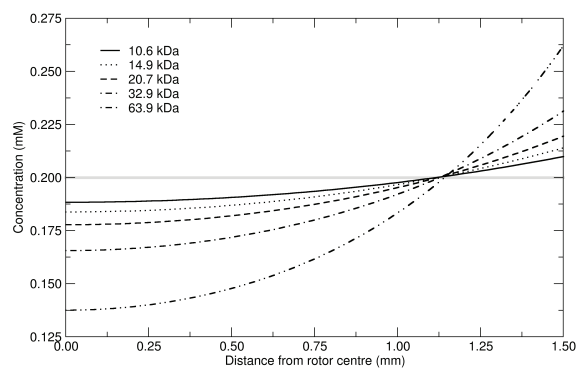
Figure 5: Concentration profiles for various PSS samples in a 4 mm (OD) HR-MAS rotor spinning at a speed (ν_r) of 2 kHz. The grey horizontal line indicates the static concentration. The curves were calculated using the method of Bertini et al.^[48, 49] from sedimentation equilibria.^[50]











Size-exclusion chromatographic NMR under HR-MAS

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Supplementary Information

The Agilent-supplied pulse sequences GCSTE (DgcsteSL.c), BPPSTE^[1] (Dbppste.c) and Oneshot^[2] (Doneshot.c) each contain two statements performing on-the-fly adjustment of the total area of the diffusion-encoding gradients. For example, the following is from Donehsot.c:

```
gt1 = syncGradTime("gt1","gzlv11",0.5);  
gzlv11 = syncGradLv1("gt1","gzlv11",0.5);
```

The first statement adjusts the length of the gradient to be an integral multiple of the rotor period, while the second corrects the power level to preserve the total area. Analysis of these experiments using Agilent's VnmrJ package or DOSY Toolbox^[3] makes use of a "dosytimecubed" parameter, calculated in the pulse sequence when the experiment is run. This parameter, the product of the gradient duration-squared and the diffusion delay (suitably corrected for the appropriate pulse sequence) is calculated using the new, corrected value of the gradient duration (gt1), however, the corrected gradient power levels (gzlv11) are not used, only the requested power levels. Figure S1 shows the result of experiments performed with the Agilent-supplied sequences and analysed with DOSY Toolbox.^[3] The result of the incorrect "dosytimecubed" parameter is the period trend in diffusion coefficient. We believe this bug is present in all of the Agilent-supplied diffusion pulse sequences, i.e. those starting D*. The simple fix is to ensure that the `syncGradTime()` and `syncGradLv1()` statements occur after the calculation of "dosytimecubed".

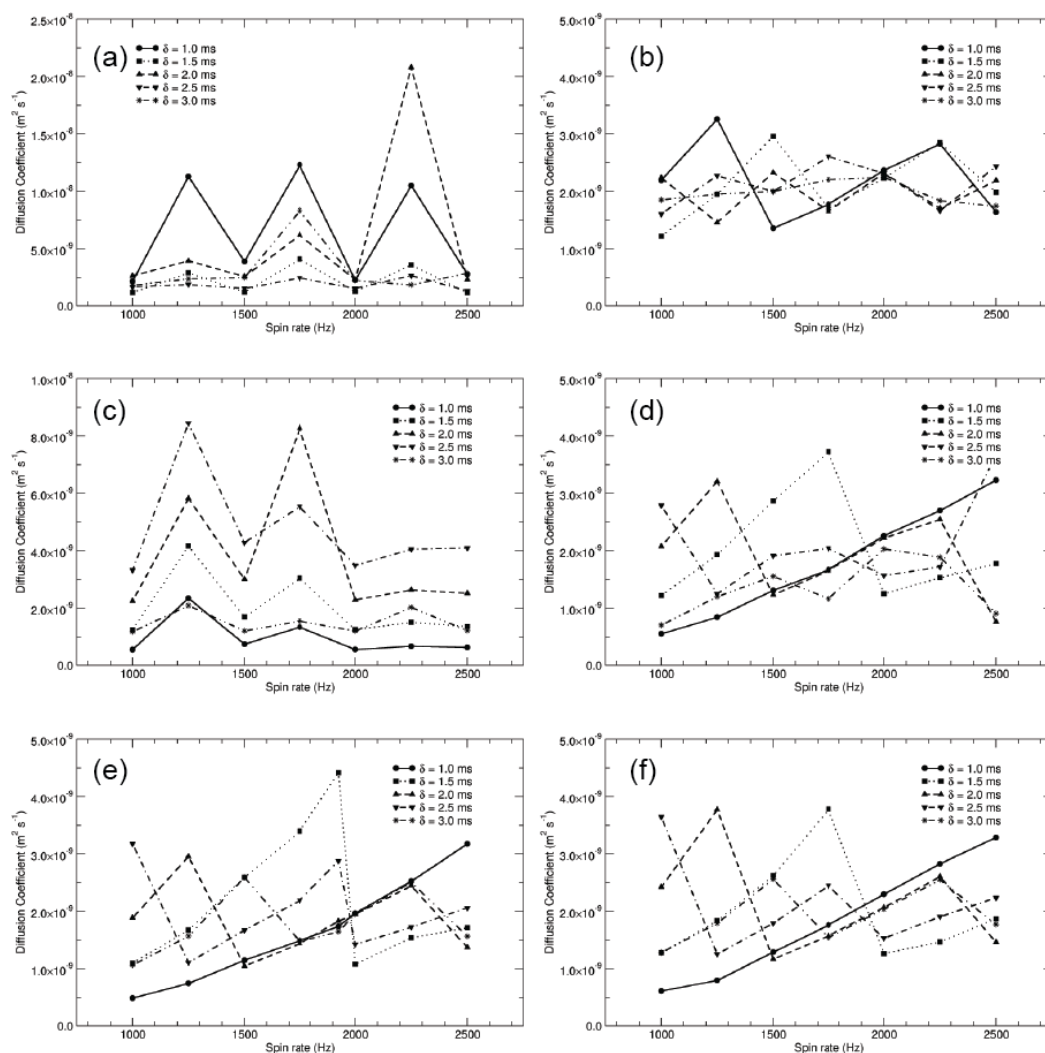


Figure S1: Observed variation in the measured diffusion coefficient of HOD with spin rate using a diffusion encoding time of Δ of 100 ms, using the Agilent-supplied pulse sequences. (a) and (b) are for the gradient compensated stimulated echo (GCSTE), (c) and (d) for the bipolar pulse stimulated echo (BPPSTE) and (e) and (f) for the Oneshot sequence. (a) (c) and (e) are the pulse sequences as supplied in the Agilent library while (b), (d) and (f) utilise complete rotor synchronisation of the RF-pulses, gradient durations and delays. The data shown in this figure is analogous to that shown in Figure 3.

References

- [1] D. H. Wu, A. D. Chen, C. S. Johnson, *J. Magn. Reson. A* **1995**, *115*, 260.
- [2] M. D. Pelta, G. A. Morris, M. J. Stchedroff, S. J. Hammond, *Magn. Reson. Chem.* **2002**, *40*, S147.
- [3] M. Nilsson, *J. Magn. Reson.* **2009**, *200*, 296.